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**Tim-3 promotes tube formation and decreases tight junction formation  
in vascular endothelial cells**

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Running title: ROLE OF TIM-3 IN ENDOTHELIAL CELLS

**Abstract.** As a negative immune checkpoint molecule, T cell immunoglobulin domain and mucin domain containing molecule-3 (Tim-3) has been found to serve a crucial role

in immune escape and tumour progression. Previous studies have reported that Tim-3 is important to endothelial cells and it has also been demonstrated to be involved in numerous types of human disease, including melanoma, lymphoma, rickettsial infection and atherosclerosis; however, its exact mechanism of action remains largely unknown. In the present study, Tim-3 was overexpressed in vascular endothelial HMVECs and HUVECs and *in vitro* assays were used to determine that Tim-3 promoted cell proliferation, migration, invasion and tube formation through activating cyclin D1, Ras homolog gene family member A and vascular endothelial growth factor receptor 2 (VEGFR2). Additionally, Tim-3 decreased tight junction (TJ) formation and the transepithelial resistance of endothelial cells by decreasing the expression levels of TJ protein 2, Occludin and claudin 1. In conclusion, these findings suggested that Tim-3 may exert a positive role in angiogenesis and a negative role in TJ formation in vascular endothelial cells, which may provide novel strategies for the treatment of Tim-3 associated diseases.

## **Introduction**

The discovery that T-cell immunoglobulin and mucin family molecules are associated with numerous types of disease, including allergy, autoimmunity [1] and cancer [2], has attracted increasing attention in recent years. In humans, the Tim family consists of T cell immunoglobulin domain and mucin domain containing molecule (Tim)-1, Tim-3 and Tim-4, which are all located on chromosome 5q33.2 [1]. Tim-3, also known as the hepatitis A virus cellular receptor 2 (HAVCR2), is an important negative immune checkpoint that serves a critical role in regulating immune cell activity [3]; it is found expressed on numerous types of immune cell, including activated T helper type 1 (Th1) cells [4], dendritic cells (DC) and monocytes/macrophages[5],[6]. Tim-3 activation has been observed to reduce T-cell-mediated cytotoxicity and is perceived as a promising target for cancer immunotherapy [7]. Moreover, Tim-3 is also overexpressed in several types of solid tumour [8],[9]and the ectopic expression of Tim-3 in tumour cells has been associated with a more advanced pathological T cell classification [10], lymph-vascular invasion [11], lung metastasis [12] and lymphatic metastasis [13]. Besides, a meta-analysis study revealed that increased expression levels of Tim-3 in solid tumours

predicted a significantly shorter overall survival [2]; thus, Tim-3 is suggested to serve as a prognostic indicator for patients.

Tim-3 is also expressed on endothelial cells [14],[15]and has been reported to serve a role in melanoma [15], lymphoma [16], rickettsial infection [17] and atherosclerosis [18],[19]; for example, Tim-3-expressing melanoma endothelial cells were found to increase the tumour cell metastatic potential through facilitating cell intravasation and extravasation [15]; Tim-3 expression in lymphoma-derived endothelial cells facilitated the growth and dissemination of lymphoma through interacting with circulating T cells and suppressing the activation of CD4<sup>+</sup> T cells; clinically, the expression levels of Tim-3 in the endothelium of B cell lymphoma were also observed to be correlated to dissemination and a poor prognosis [16]; and increased expression levels of Tim-3 facilitated intracellular rickettsial killing in endothelial cells during the early phase of rickettsial infection [17]. In addition, Tim-3 was demonstrated to act as a negative regulator of atherosclerosis, which was accompanied by increased levels of circulating monocytes and lesional macrophages and decreased levels of regulatory T cells and regulatory B cells [18]. Tim-3 also protected HUVECs from ox-low-density lipoprotein (LDL)-induced apoptosis via the c-Jun N-terminal kinase (JNK) pathway and reversed the inhibitory effect over migration [19]. Tim-3 was also revealed to inhibit ox-LDL-induced inflammatory cytokine production; however, this was due to its suppression over NF-κB activation [19]. Overall, these findings suggested that Tim-3 may serve an important role in endothelial-related diseases in humans.

Our previous study revealed that the overexpression of HAVCR-1 (Tim-1) resulted in reduced tight junction (TJ) formation in human endothelial cells by co-localising with Zonula occludens proteins including ZO-1 and ZO-2 [20]. Therefore, in the present study, the effects of Tim-3 on cell proliferation, migration, invasion and tube formation were investigated in human lung microvascular endothelial cells (HMVECs) and human umbilical vein endothelial cells (HUVECs), and subsequently, the potential role of Tim-3 in TJs was analysed.

## **Materials and methods**

### **Cell lines and culture**

HMVECs (Catalog #: CC-2543) and HUVECs (Catalog #: C2519A) were purchased from Lonza (Slough, UK). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 with L-Glutamine (Sigma-Aldrich; Merck KGaA), supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA) and antibiotics (Sigma-Aldrich; Merck KGaA). Cells were maintained at 37°C and 5% CO<sub>2</sub> in a humidified incubator.

### **Establishment of stable cell lines overexpressing Tim-3**

To establish Tim-3 overexpressing endothelial cells, the full length of Tim-3 (PLV [Exp]-EGFP: T2A: Puro-CMV> hHAVCR2 [NM\_032782.4]) or negative control scramble (PLV [Exp]-EGFP: T2A: Puro-CMV>stuffer\_300bp) was transfected into HMVECs and HUVECs using a lentivirus vector (VectorBuilder Inc.). Briefly, 5x10<sup>4</sup> cells were seeded into a 6-well plate and medium containing 10 µg/ml polybrene and lentiviral vector was added the following day. The medium was removed and replaced with normal medium following incubation for 20 h. A total of 2 µg/ml puromycin (Sigma-Aldrich; Merck KGaA) was used following culture for 3 days to select the stable cell lines. Following the selection, the cells were cultured in normal medium containing 0.25 µg/ml puromycin.

### **Reverse transcription-quantitative PCR (RT-qPCR)**

Total RNA was extracted from cultured cells using TRIzol<sup>®</sup> reagent (Sigma-Aldrich; Merck KGaA) and was reverse transcribed into cDNA using the GoScript<sup>™</sup> Reverse Transcription System kit (Promega Corporation)[21], according to the manufacturer's protocol. qPCR was subsequently performed using an iCycler iQ<sup>™</sup> (Bio-Rad Laboratories, Inc.). The following thermocycling conditions were used for the qPCR: 94°C for 5 min; and 100 cycles of 94°C for 10 sec, 55°C for 35 sec and 72°C for 20 sec. The following primer sequences used are presented in Table I. All the primers were synthesised by Sigma-Aldrich (Irvine, UK). They were dissolved using dd H<sub>2</sub>O to 100 µM and stored at -20°C. mRNA expression levels were quantified using the 2<sup>-ΔΔC<sub>q</sub></sup> method and normalized to GAPDH.

### **Western blotting**

Total protein was extracted from cells using a protein lysis buffer. Total protein was quantified using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Inc.) and equal amounts of protein were separated via SDS-PAGE. The separated proteins were

subsequently transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with 5% skimmed milk for 2 h. The membranes were incubated with the following primary antibodies: Anti-Tim-3 antibody (cat. no. ab241332; Abcam), anti-GAPDH (cat. no. sc-47724; Santa Cruz Biotechnology, Inc.), anti-cyclin D1(CCND1) (cat. no. sc-8396; Santa Cruz Biotechnology, Inc.), anti-ZO2 (cat. no. sc-11448; Santa Cruz Biotechnology, Inc.) and anti-Occludin (cat. no. sc-133256; Santa Cruz Biotechnology, Inc.). Following the primary antibody incubation, the membranes were incubated with anti-mouse (cat. no. A5278; Sigma-Aldrich; Merck KGaA), anti-rabbit (cat. no. A0545; Sigma-Aldrich; Merck KGaA) and anti-goat (cat. no. A8919; Sigma-Aldrich; Merck KGaA) secondary antibodies. Protein bands were visualized using Luminata Forte (Merck KGaA) and quantified using ImageJ software (National Institutes of Health) based on the band intensities.

### **Cell proliferation assay**

Cell proliferation was assessed using the AlamarBlue assay. Briefly,  $2 \times 10^3$  cells/well were seeded into a 96-well plate and incubated for 6 days; the medium was replaced every 3 days during this period. Following incubation for 2, 4 and 6 days, the medium (15 wells per group) was aspirated and 100  $\mu$ l fresh medium, containing 10  $\mu$ l AlamarBlue (Bio-Rad Laboratories, Inc.) was added to each well and incubated for 3 h at 37°C. The fluorescence was detected using a fluorescence plate reader (Promega Corporation), using an excitation wavelength of 525 nm and an emission wavelength of 590 nm. The percentage growth following the incubation period was then calculated against the overnight plates.

### **Wound healing assay**

A total of  $2 \times 10^5$  cells were seeded in a 24-well plate and cells were subsequently scratched using a 1 ml pipette tip to generate an artificial wound in the cell monolayer. After washed the cells twice with PBS, normal medium was added to each well. The migratory ability was monitored using an EVOS<sup>®</sup> FL imaging system (Thermo Fisher Scientific, Inc.) at a 4X objective every 2 h for 24 h. The percentage wound closure at the experimental endpoint compared to at 0 h was analysed using ImageJ software.

### **Matrigel invasion assay**

The cell invasive ability was assessed using an *in vitro* Matrigel invasion assay. Briefly, Transwell inserts (8- $\mu\text{m}$  pores) for 24-well plates were precoated with 100  $\mu\text{l}$ /insert 0.5mg/ml Matrigel (BD Biosciences) for 1 h at 37°C. Subsequently, a total of  $2 \times 10^4$  cells were plated in the upper chambers of Transwell plates in 150  $\mu\text{l}$  DMEM. A total of 650  $\mu\text{l}$  normal medium was plated in the lower chambers. Following incubation for 48 h, non-invasive cells remaining in the upper chambers were removed with a cotton swab. The invasive cells in the lower chambers were fixed with 4% formalin for 30 min and stained with 1% crystal violet for 30 min, before rinsing with phosphate-buffered saline (PBS). Stained cells were counted under a microscope with  $\geq 5$  counts per experimental setting.

#### **Cell-matrix adhesion assay**

A 96-well plate containing Matrigel (10  $\mu\text{g}$ /well) was incubated at 37°C for 2 h. A total of  $2 \times 10^4$  cells/well were added and incubated for 1 h and then washed twice with PBS. Adhesive cells were fixed with 4% formalin and stained with 1% crystal violet before rinsing with PBS. The number of attached cells was counted under a microscope with  $\geq 5$  counts per experimental setting.

#### **Tube formation assay**

Prechilled 96-well plates were coated with 50  $\mu\text{l}$ /well Matrigel (BD Biosciences) and incubated to polymerize at 37°C for 1 h. A total of  $2 \times 10^4$  cells were plated into each well and incubated at 37°C and 5%  $\text{CO}_2$  for 16 h. Five views from five wells of each group were then captured to evaluate the tube formation ability by counting the total segments length automatically using ImageJ software. A segment was defined as an element delimited by two junctions of the newly formed tubule network.

#### **Electric cell-substrate impedance sensing (ECIS) assay**

The ECIS Z $\theta$  system with 96W1E+ array plate (Applied BioPhysics, Inc.) was used to measure the initial attachment and spreading of cells. Briefly, the plate was stabilized using normal medium for 2 h and  $5 \times 10^4$  cells/well were seeded and cultured for 24 h. The resistance across the array was recorded at different frequencies.

#### **Transepithelial resistance (TER) and paracellular permeability (PCP) assays**

An EVOM Voltohmmeter (World Precision Instruments), equipped with STX2 chopstick electrodes (World Precision Instruments) was used to measure the TER. Briefly,  $5 \times 10^4$

cells were plated into a 0.4- $\mu$ m pore size insert (Greiner Bio-One Ltd) and cultured to 100% confluence. Electrodes were placed in the upper and lower chambers and resistance was subsequently measured using a Volt-Ohm meter. Inserts without cells in medium were set as a blank control. Following the analysis of TER, the medium in the upper chambers was replaced with normal medium containing 0.2mg/ml FITC (Fluorescein isothiocyanate)-dextran 10 kDa. Then, 50  $\mu$ l medium from outside of the insert was transferred into a black 96-well cell culture microplate (Greiner Bio-One) in duplicate every 2 h for 10 h. The basolateral dextran passage was analysed using a GloMax<sup>®</sup>-Multi Microplate Multimode reader (Promega Corporation), with an excitation wavelength of 490 nm and an emission wavelength of 510-570 nm. Each measurement was normalized to the 0 h via subtraction.

### **Enzyme-linked immunosorbent assay (ELISA)**

Cultured cells were concentrated using the Amicon Ultra-4 centrifugal filter units (Sigma-Aldrich; Merck KGaA) and the medium was subsequently used for ELISA. ELISA was performed using the human vascular endothelial growth factor receptor 2 (VEGFR-2) ELISA kit (cat. no. E-EL-H1603; Elabscience), according to the manufacturer's protocol.

### **Bioinformatical analysis**

The transcription level correlation of Tim-3 with angiogenic regulators was investigated using a pooled analysis of publicly available endothelial (single) cell gene expression data from the EndoDB Version 05/07/2019 (<https://endotheliomics.shinyapps.io/endodb/>). Only studies on primary or freshly isolated endothelial cells were selected, and untreated or control groups were recruited, which provided a cohort with 643 samples. The correlation coefficients (R) were measured using Pearson's correlation method.

### **Immunohistochemistry (IHC) assay**

Breast cancer tissues were collected after surgical resection at the Affiliated Yantai Yuhuangding Hospital of Qingdao University with written consent forms from each patient. The protocol was approved by the local Research Ethics Committee. The IHC assay was performed following our standard operation protocol using a Tim-3 antibody (ab241332. Abcam, Cambridge, UK).

### **Statistical analysis**



Statistical analysis was performed using GraphPad Prism version 7.0 software (GraphPad Software, Inc.) and data are presented as the mean  $\pm$  SD. For the ECIS data with repeated measures (RM) of time-lapse, statistical differences between two groups were analysed using two-way ANOVA with RM and multiple comparisons. The post-hoc test following the repeated measures ANOVA was Tukey's multiple comparisons test. For other experimental data, group comparison was performed using a two-sided Student's t-test when the data were normally distributed or Mann-Whitney U test when the data were not normally distributed. Experiments were repeated 2-4 times unless otherwise stated. The number of samples per group in each experiment was at least three (n=3) if not indicated. P<0.05 was considered to indicate a statistically significant difference. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001.

## **Results**

### **Stable endothelial cell lines overexpressing Tim-3**

HMVECs and HUVECs were stably transfected with full-length Tim-3 overexpression plasmid or empty vector [Scramble (Scr)] and the expression levels of Tim-3 in the two cell lines were analysed using RT-qPCR and western blotting. Tim-3 expression levels were significantly increased in Tim-3 overexpression (Tim-3 OE) cells compared with the Scr and wild-type cells at both the mRNA and protein level (Fig. 1A), which confirmed that the Tim-3 overexpression plasmid was successfully transfected to the cells.

### **Tim-3 promotes cell proliferation and adhesion *in vitro***

The effect of Tim-3 expression on cell proliferation and adhesion *in vitro* was subsequently investigated. Cell proliferation was significantly increased in both Tim-3 OE HMVECs and HUVECs compared with the Scr cells when cultured for 4 or 6 days (all P<0.001; Fig. 1B and 1C). The adhesive ability of cells was assessed using a cell-matrix adhesion assay; it was observed that the adhesive ability was also increased when Tim-3 expression levels were overexpressed in both cell lines (all P<0.001; Fig.1D and 1E).

CCND1 is an important molecule in cell proliferation, thus it was subsequently investigated whether CCND1 was regulated when Tim-3 was overexpressed. CCND1 expression levels were increased in both Tim-3 OE HMVECs and HUVECs at the protein

level (Fig. 1F and 1G), which may partially explain the stimulatory role of Tim-3 in cell proliferation.

### **Tim-3 promotes cell migration and invasion *in vitro***

A Matrigel invasion assay and wound healing assay were used to characterise the effects of Tim-3 on cell invasion and migration. The Matrigel invasion assay revealed that Tim-3 OE cells were more invasive compared with Scr cells in both HMVECs (P=0.033; Fig. 2A) and HUVECs (P=0.030; Fig. 2B). Similarly, the wound healing assay demonstrated that the cell migration ability was also increased in Tim-3 OE HMVECs at 24 h (P<0.001; Fig. 2C) and HUVECs at 24 h (P=0.026; Fig. 2D).

Ras Homolog Family Member A (RhoA) is a critical molecule in the regulation of cell migration, thus it was subsequently investigated whether RhoA was involved in the promotion of cell migration. RhoA was significantly increased when Tim-3 expression levels were overexpressed in both HMVECs and HUVECs (Fig. 1F and 1G).

### **Tim-3 promotes tube formation *in vitro***

The formation of capillary-like tubes by endothelial cells on a membrane matrix is a powerful method to evaluate the capacity of angiogenesis. The tube formation ability was significantly increased in Tim-3 OE HMVECs compared with Scr cells when cultured for 8 h (P=0.005) and 16 h (P=0.001; Fig. 3A). A similar phenomenon was also observed in Tim-3 OE HUVECs when cultured for 8 h (P=0.015; Fig. 3B), which suggested that the overexpression of Tim-3 may have a potential promoting role over angiogenesis in endothelial cells.

To investigate the mechanisms by which Tim-3 promotes tube formation, a VEGFR2 ELISA was performed using the Tim-3 OE cells and their respective Scr controls. The stable expression of Tim-3 in both HMVECs and HUVECs resulted in increased expression levels of VEGFR2 protein (P<0.05; Fig. 3C). Besides, bioinformatics analysis using EndoDB revealed that Tim-3 was significantly correlated with the transcription of Angiogenin (ANG), an angiogenic protein (Fig. 3D).

### **Tim-3 reduces the integrity of endothelial TJs**

TJs serve a crucial role in the regulation of endothelial barrier functions and their disruption usually leads to increased paracellular permeability. Thus, the role of Tim-3 in

TJs was investigated. ECIS was firstly used to investigate the initial cell attachment and spreading by monitoring the resistance at 1 kHz; a lower resistance was observed in Tim-3 OE cells compared with Scr cells during the initial attachment and spreading in both HMVECs and HUVECs (Fig. 4A), which indicated that Tim-3 may decrease the integrity of cell-cell TJs.

To further confirm our primary findings, TER and PCP assays were performed to investigate the effect of Tim-3 on the TJ barrier function. The TER in Tim-3 OE cells was decreased compared with the control cells in both HMVECs and HUVECs (all  $P < 0.001$ ; Fig. 4B), which suggested that Tim-3 overexpression may reduce the cell-to-cell connectivity. Following determination of the TER, PCP FITC-dextran 10 kDa was used to evaluate the permeability between cells monolayers; however, no significant difference was observed between Tim-3 OE cells and Scr cells in both cell lines.

Based on the above findings, the mechanism of Tim-3 in TJs was investigated. From our results, ZO-2, Occludin and claudin 1 (CLDN1) expression levels were all discovered to be decreased in both Tim-3 OE HMVECs and HUVECs, as determined using western blotting (Fig. 1F and 1G), which may partially explain the negative role of Tim-3 in TJs.

### **Tim-3 is highly expressed by vascular endothelial cells in breast cancer tissue**

The cultured human endothelial cells may have a different microenvironment to mediate the expression of Tim-3. We hypothesized that the Tim-3 expression could be altered under certain pathological conditions such as cancer. To examine this we evaluated the expression levels of Tim-3 protein in breast cancer tissue sections by IHC. As shown in Fig. 5, Tim-3 was expressed by the vascular endothelial cells in the breast cancer tumour sections with high frequency. This confirmed that there is protein expression of Tim-3 in endothelium *in vivo* at least when malignancy occurred.

### **Discussion**

Endothelial cells form a one-cell thick monolayer known as the endothelium, which lines the interior surface of blood vessels. Previous studies have demonstrated that the expression of Tim-3 on endothelial cells was associated with several vascular-associated diseases. Therefore, in the present study, Tim-3 was overexpressed in vascular endothelial cells, and *in vitro* functional assays were used to investigate the potential roles and mechanisms of Tim-3.

One of the main functions of the endothelium is to form new blood vessels through a process known as angiogenesis. Angiogenesis has important applications in cancer research, because tumour growth is supported by the creation of new blood vessels. Therefore, current research is focused on inhibiting the process of angiogenesis to prevent tumour progression. During angiogenesis, dynamic changes in the adhesive structures and mobility are crucial for cellular remodelling. In the present study, the growth of Tim-3 overexpressing cells was significantly enhanced in both HMVECs and HUVECs through activating CCND1. CCND1 promotes cell growth through forming active complexes with either cyclin-dependent kinase 4 (CDK4) or CDK6, which in turn phosphorylates the retinoblastoma protein (Rb) and drives the progression from the G1 to S phase [22]. Tim-3 has also been observed to promote cell proliferation through activating the NF- $\kappa$ B signalling pathway in B16 melanoma endothelial cells [15]. The migratory and invasive abilities of cells overexpressing Tim-3 was subsequently investigated and it was discovered that Tim-3 overexpression could increase the cell migratory and invasive ability in both HMVECs and HUVECs. In B16 melanoma cells, endothelial cell-expressed Tim-3 was reported to increase the cell metastatic potential through facilitating tumour cell intravasation, survival in blood stream and extravasation [15], which supported our results. Cell migration is a multi-step process, which serves an important role in numerous types of diseases. An important regulatory element of cell migration is the interdependent regulation of ras-related C3 botulinum toxin substrate 1 (Rac1) and Rho; Rac1 is required at the front of the cell to regulate actin polymerization and membrane protrusion, whereas Rho appears to regulate the contraction and retraction forces required in the cell body and at the rear [23]. Thus, the current study explored whether RhoA was involved in Tim-3-induced cell migration. Unsurprisingly, RhoA was increased in Tim-3 overexpressing HMVECs and HUVECs, which may partially explain its role in promoting cell migration.

Since Tim-3 was found to exert a promotive role in cell growth, migration and invasion, which are crucial mechanisms involved in angiogenesis, it was subsequently determined whether Tim-3 could promote tube formation *in vitro*. As illustrated from our results, the upregulation of Tim-3 also increased the tube-forming ability, which suggested that Tim-3 may serve a positive role in angiogenesis. The underlying mechanism by which Tim-3 promotes tube formation may be due to its ability to induce VEGFR production, which

therefore enhances the regulation of vascular endothelial growth factor (VEGF); however, Tim-3 may also mediate tube formation in a VEGF/VEGFR independent manner, such as through the ANG signalling pathway.

In a recent study, another costimulatory molecule, B7 homolog 3 protein (B7-H3), was also demonstrated to increase cell proliferation, migration and tube formation in HUVECs through promoting VEGF secretion [24]. The vascular endothelium in the tumour microenvironment is essential in tumour progression, thus the fact that Tim-3 can promote angiogenesis may suggest its significance in tumour progression.

Another significant function of the endothelium is to maintain homeostasis through regulating the permeability of vessels. This property is well researched in relation to the blood-brain-barrier system due to the difficulty in developing drugs that can cross the endothelial barrier efficiently. Current research is focused on better understanding the functions of the blood brain barrier system. A previous study reported that HAVCR-1 (Tim-1) could downregulate the apparatus required for TJ formation in human endothelial cells by co-localising with ZO-1 and ZO-2 proteins, which reduced TJ formation [20]. Therefore, TJ function was also investigated in the present study to determine whether Tim-3 was also involved in TJs. ECIS was firstly used to investigate the initial attachment and spreading; a lower resistance was observed in Tim-3 OE cells compared with Scr cells in both HMVECs and HUVECs, which implied that Tim-3 may decrease the integrity of TJs. Subsequently, the TER assay further validated these findings, as a decreased TER was found in Tim-3 OE cells compared with Scr cells in both cell lines; however, no significant difference was found in the monolayer cell permeability between the Scr and Tim-3 OE cells, which was determined using the PCP assay with FITC-dextran 10 kDa. It was suggested that this may be due to the molecular weight (10 kDa) used in our detection.

Important TJ molecules were subsequently analysed to determine the mechanism of Tim-3 in TJs. From our results, ZO-2, Occludin and CLDN1 expression levels were all decreased in both Tim-3 OE HMVECs and HUVECs. ZO-2 is a peripheral TJ protein belonging to the membrane-associated guanylate kinase protein family. Occludin has been reported to directly bind to N-ZO-2, as well as the NH<sub>2</sub>-terminal Discs-large-like portion of ZO-1 (N-ZO-1) *in vitro* [25]. Previously, in ZO-1 KO/ZO-2 KD epithelial cells,

no TJs were found, although cells retained a polarized distribution of membrane proteins [26]. Therefore, it was hypothesised that Tim-3 may decrease TJ functions through downregulating ZO-2 expression, which would further downregulate Occludin and CLDN1 expression levels; however, the mechanism by which Tim-3 downregulates TJs requires further research in the future.

Migration of vascular endothelial cells is critical in maintaining the integrity of blood vessel walls and leads to the formation of new blood vessels, thereby improving the prognosis of atherosclerosis [27]. Inhibition of vascular endothelial cell migration promotes atherosclerosis progression through suppressing the repair of damaged vascular endothelial cells and angiogenesis. Tim-3 promotes cell proliferation and migration in vascular endothelial cells, suggesting its potential in the treatment of atherosclerosis [19]. We demonstrate that there is an endogenous expression of Tim-3 protein in the vascular endothelial cells from breast cancer tissue. Tim-3 might be upregulated on vascular endothelium in patients with tumour malignancies due to the stimulation by growth factors in the tumour microenvironment. Tim-3 could promote tube formation and downregulate TJs *in vitro*, indicating that Tim-3 may play a great role in cancer invasion and metastasis through inducing angiogenesis and increasing capillary permeability. Further investigation will be encouraged though to obtain a deeper insight in the future.

As one of the limitations of this study, it may be worthy to be noted that Tim-3 knockdown *in vitro* models was not established to check whether Tim-3 knockdown has the opposite function. Future work will be encouraged to estimate the role of Tim-3 in angiogenesis in particular in pathological conditions using an appropriate animal model.

In conclusion, the proliferative, adhesive, migratory and invasive ability were all increased when Tim-3 expression levels were overexpressed in HMVECs and HUVECs through the activation of CCND1 and RhoA. Tim-3 also served a positive role in promoting tube formation and reducing cell-cell TJs and transepithelial resistance through decreasing ZO-2, Occludin and CLDN1 expression levels.

### **Abbreviations**

Tim-3, T cell immunoglobulin domain and mucin domain containing molecule-3;

VEGFR2, vascular endothelial growth factor receptor 2; TJ, tight junction; HAVCR2,

hepatitis A virus cellular receptor 2; Th1, T helper type 1; DC, dendritic cells; LDL, low-density lipoprotein; JNK, Jun N-terminal kinase; ZO-1, zonula occludens-1; ZO-2, zonula occludens-2; HMVEC, human lung microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; DMEM, Dulbecco's Modified Eagle's medium; PVDF, polyvinylidene difluoride; CCND1, Cyclin D1; PBS, Phosphate-buffered saline; ECIS, Electric cell-substrate impedance sensing; TER, Transepithelial resistance; PCP, paracellular permeability; FITC, Fluorescein isothiocyanate; IHC, Immunohistochemistry; RM, repeated measures; CLDN1, Claudin 1; ANG, Angiogenin; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; Rb, retinoblastoma protein; Rac1, Ras-related C3 botulinum toxin substrate 1; RhoA, Ras Homolog Family Member A; VEGF, Vascular endothelial growth factor; B7-H3, B7 homolog 3 protein.

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### **Availability of data and material**

All data relevant for this study are given with the main paper including figures and tables. The primary data that support the findings of this study are available from the corresponding author upon reasonable request.

### **Author contributions**

YZC, XMW, YXC and WGJ conceived and planned the experiments. YZC and JQC carried out the experiments. XMW and GDQ contributed to the interpretation of the results. YZC and YLL took the lead in writing the manuscript in consultation with YXC

and WGJ. All authors provided critical feedback and helped shape the research, analysis and manuscript.

### **Ethics approval and consent to participate**

Approval for this study was obtained from the Yantai Yuhuangding Hospital Human Ethical Committee (reference number: 2018-109). This study was performed in accordance with the Helsinki declaration.

### **Competing interests**

The authors declare no conflict of interest.

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Figure 1. Stable HMVECs and HUVECs overexpressing Tim-3 were established, which was found to increase cell proliferation and adhesion *in vitro*. (A) Successful overexpression of Tim-3 in HMVECs and HUVECs was analysed using reverse transcription-quantitative PCR and western blotting. (B and C) Overexpression of Tim-3 increases cell proliferation in (B) HMVECs and (C) HUVECs. (D and E) Overexpression of Tim-3 enhances the cell adhesive ability of (D) HMVECs ( $P < 0.001$ ) and (E) HUVECs ( $P < 0.001$ ). (F) Protein expression levels of CCND1, RhoA, ZO-2, Occludin and CLDN1 in Scr and Tim-3 OE cells was analysed using western blotting. Following normalization of the cell lysate samples, the Western blots were derived from different membranes with equal-amount sample loading and GAPDH blots as a control. (G) Semi-quantitative analysis of western blotting using ImageJ software. (H) Gene expression levels of CCND1, RhoA, ZO-2, Occludin and CLDN1 were determined using reverse transcription-quantitative PCR.

Figure 2. Overexpression of Tim-3 increases cell invasion and migration *in vitro*. (A and B) Overexpression of Tim-3 increases the invasive ability of (A) HMVECs ( $P = 0.033$ ) and (B) HUVECs ( $P = 0.030$ ). (C and D) Wound healing assay was used to investigate the cell migration ability, which was found to be increased in Tim-3 OE (C) HMVECs (24 h;  $P < 0.001$ ) and (D) HUVECs (24 h;  $P = 0.026$ ).

Figure 3. Overexpression of Tim-3 promotes tube formation in vascular endothelial cells. (A and B) Tube formation ability is increased in Tim-3 OE (A) HMVECs (8 h;  $P = 0.005$ ; 16 h;  $P = 0.001$ ) and (B) HUVECs (8 h;  $P = 0.015$ ). (C) VEGFR2 expression levels were analysed using ELISA. (D) Correlation analysis between Tim-3 transcriptional levels and angiogenic factors. Endothelial (single) cell gene expression data were extracted from the EndoDB Version 05/07/2019 (<https://endotheliomics.shinyapps.io/endodb>).

Figure 4. Overexpression of Tim-3 decreases cell-cell tight junctions in vascular endothelial cells. (A) Overexpression of Tim-3 promotes a slower initial attachment and spreading of HMVECs and HUVECs, which was assessed using ECIS. (B) Overexpression of Tim-3 decreases the TER in both HMVECs ( $P < 0.001$ ) and HUVECs ( $P < 0.001$ ). (C) Overexpression of Tim-3 did not affect the permeability (FITC-dextran 10 kDa) between cells monolayers in HMVECs and HUVECs.

Figure 5. In-vivo expression of Tim-3 protein in breast cancer tissue indicated by an IHC assay. (A) No primary antibody control. (B) Representative vascular endothelium that is negative for Tim-3. (C-D) Representative vascular endothelia that are positive for Tim-3. The representative vascular endothelium sites were highlighted using red arrows.

